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FFPE RNA Purification 96-Well Kit — Product Insert

Product # 25400

Norgen's FFPE RNA Purification Kit provides a rapid method for high throughput isolation and purification of total RNA (including microRNA) from formalin-fixed paraffin-embedded (FFPE) tissue samples. Using formalin to fix tissues leads to crosslinking of the RNA and proteins, and the process of embedding the tissue samples can also lead to fragmentation of the RNA over time. Norgen's FFPE RNA Purification Kit provides conditions that allow for the partial reversing of the formalin modifications, resulting in a high quality and yield of RNA. The kit is able to purify all sizes of RNA, from large mRNA and ribosomal RNA down to microRNA (miRNA) and small interfering RNA (siRNA), depending on the age of the FFPE tissue as the degree of fragmentation of the RNA will increase over time. The RNA is preferentially purified from other cellular components without the use of phenol or chloroform. The purified RNA is of the highest integrity, and can be used in a number of downstream applications including qRT-PCR, reverse transcription PCR, primer extension, expression array assays, and microarray analyses.

Norgen's Purification Technology

Purification is based on 96-well column chromatography using Norgen's proprietary resin as the separation matrix. The RNA is preferentially purified from other cellular components without the use of phenol or chloroform. The process first involves deparaffinization of the FFPE samples through a series of xylene and ethanol washes. Next, the FFPE samples are digested with the provided Proteinase K and Digestion Buffer. Binding Solution and ethanol are then added to the lysate, and the solution is loaded onto the 96-Well Filter Plate for purification. The purification could be performed on either a vacuum manifold or using centrifugation. Norgen's resin binds nucleic acids in a manner that depends on ionic concentrations, thus only the RNA will bind to the resin while other contaminants will be removed in the flowthrough or retained on the top of the resin. At this point, any remaining traces of genomic DNA can be digested using an optional protocol, allowing for pure RNA samples to be isolated. The bound RNA is then washed with the provided RNA Wash Solution in order to remove any impurities, and the purified total RNA is eluted with the Elution Solution.

Specifications

Kit Specifications	
Maximum Binding Capacity per Well	50 µg
Maximum Loading Volume per Well	500 µL
Size of RNA Purified	All sizes, including small RNA (<200 nt)
Maximum Amount of Starting Material	5 sections ≤20 µM thick 25 mg of unsectioned block

Advantages

- Fast and easy processing using either a vacuum manifold or centrifugation
- High yields of total RNA
- Isolate total RNA, from large rRNA down to microRNA (miRNA)
- No phenol or chloroform extractions

Storage Conditions and Product Stability

All solutions should be kept tightly sealed and stored at room temperature. The DNAse I should be stored at -20°C upon arrival. The Proteinase K should be stored at -20°C upon arrival and after reconstitution. These reagents should remain stable for at least 1 year in their unopened containers.

Kit Components

Component	Product # 25400 (2 x 96 preps)
Digestion Buffer	2 x 25 mL
Binding Solution	2 x 25 mL
Enzyme Incubation Buffer	2 x 8 mL
Wash Solution	2 x 30 mL
Elution Solution	2 x 20 mL
Proteinase K	2 x 20 mg
DNase I	2 x 500 µL
96-Well Incubation Plate	2
96-Well Filter Plate	2
Adhesive Tape	8
96-Well Collection Plate	2
96-Well Elution Plate	2
Product Insert	1

Precautions and Disclaimers

This kit is designed for research purposes only. It is not intended for human or diagnostic use. Ensure that a suitable lab coat, disposable gloves and protective goggles are worn when working with chemicals. For more information, please consult the appropriate Material Safety Data Sheets (MSDSs). These are available as convenient PDF files online at *www.norgenbiotek.com*.

Blood of all human and animal subjects is considered potentially infectious. All necessary precautions recommended by the appropriate authorities in the country of use should be taken when working with whole blood.

The **Binding Solution** contains guanidine salts, and should be handled with care. Guanidine salt forms highly reactive compounds when combined with bleach, thus care must be taken to properly dispose of any of these solutions.

Customer-Supplied Reagents and Equipment

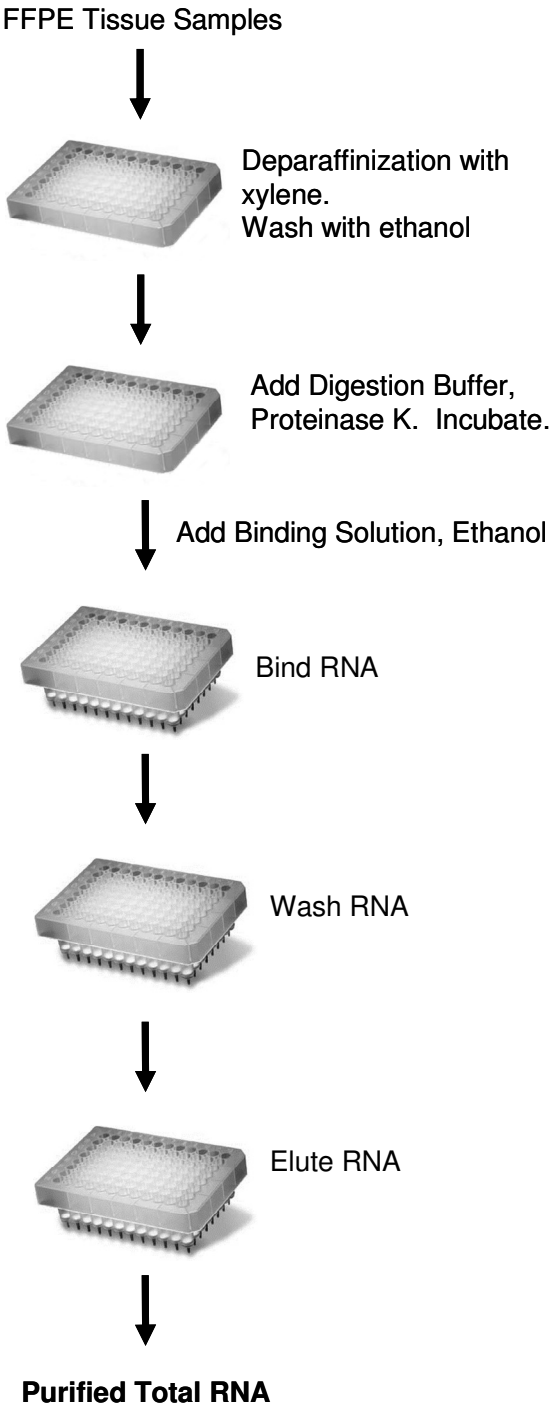
You must have the following in order to use the FFPE RNA Purification Kit:

For All Protocols

- For ***Vacuum Format Purification***:
  - Vacuum manifold with vacuum pump capable of generating a minimum pressure of -650 mbar or -25 in. Hg (such as Whatman UniVac 3 Vacuum to Collect Manifold)
  - Sealing tape or pads
- For ***96-Well Centrifuge Format Purification***:
  - Centrifuge with rotor for 96-well plate assembly (such as Thermo Fisher IEC Centra CL3 series or Beckman GS-15R)
- 95 - 100% ethanol
- Xylene, histological grade
- Incubators set at 55°C and 80°C
- β-mercaptoethanol (optional)
- RNase-Free Microcentrifuge Tubes (optional)
- Collection/Waste Tray for vacuum manifold or 96-well bottom plate (single or 96-well format) for centrifugation. Two 96-Well Collection Plates are provided with the kit.

Flowchart

Procedure for Purifying Total RNA using Norgen's FFPE RNA Purification 96-Well Kit



Working with RNA
RNases are very stable and robust enzymes that degrade RNA. Autoclaving solutions and glassware is not always sufficient to actively remove these enzymes. The first step when preparing to work with RNA is to create an RNase-free environment. The following precautions are recommended as your best defense against these enzymes.

- The RNA area should be located away from microbiological work stations
- Clean, disposable gloves should be worn at all times when handling reagents, samples, pipettes, disposable tubes, etc. It is recommended that gloves are changed frequently to avoid contamination
- There should be designated solutions, tips, tubes, lab coats, pipettes, etc. for RNA only
- All RNA solutions should be prepared using at least 0.05% DEPC-treated autoclaved water or molecular biology grade nuclease-free water
- Clean all surfaces with commercially available RNase decontamination solutions
- When working with purified RNA samples, ensure that they remain on ice during downstream applications

Procedures

For *Vacuum Manifold*: All vacuum steps are performed at room temperature. The correct pressure can be calculated using the conversions:

1 **mbar** = 100 **Pa** = 0.0394 **in. Hg** = 0.75 **mm Hg** = 0.0145 **psi**

For *Centrifugation*: All centrifugation steps are performed at room temperature. The correct rpm can be calculated using the formula:

$$RPM = \sqrt{\frac{RCF}{(1.118 \times 10^{-5})\ (r)}}$$

where *RCF* = required gravitational acceleration (relative centrifugal force in units of g); *r* = radius of the rotor in cm; and *RPM* = the number of revolutions per minute required to achieve the necessary *g*-force.

Notes Prior to Use

- Ensure that all solutions are at room temperature prior to use.
- All enzymes provided should remain at the storage temperature indicated on each vial until use.
- Reconstitute the **Proteinase K** in 1 mL of molecular biology grade water, aliquot into small fractions and store the unused portions at -20°C until needed.
- Prepare a working concentration of the **Wash Solution** by adding 90 mL of 95% ethanol (provided by the user) to the supplied bottle containing the concentrated **Wash Solution**. This will give a final volume of 120 mL. The label on the bottle has a box that may be checked to indicate that the ethanol has been added.
- **Optional:** For sensitive downstream applications or target transcripts of low quantity, add 10 µL of β-mercaptoethanol (provided by the user) to each 1 mL of **Binding Solution** required. β-mercaptoethanol is toxic and should be dispensed in a fume hood. Alternatively, the **Binding Solution** can be used as provided.
- The maximum recommended input is five sections of ≤ 20 µm thick. Alternatively, an unsectioned block of up to 25 mg may be used.
- It is important to obtain sections from the interior of an FFPE block in order to minimize RNA damage by oxidation.
- It is important to work quickly during this procedure.

Section 1. FFPE Tissue Deparaffinization and Lysate Preparation

Note: Deep-well 96-Well Incubation Plates are provided with the kit for high throughput preparation of lysate from FFPE samples (**Step 1A**). Alternatively, the user may use RNase-free microcentrifuge tubes (not provided) for lysate preparation (**Step 1B**).

1A. Deparaffinization using Provided 96-Well Incubation Plate

a. Cut sections up to 20 µm thick from the interior of an FFPE tissue block using a microtome. Trim off any excess paraffin.

   Note: Alternatively, cut out up to 25 mg of unsectioned core from an FFPE block. Trim off any excess paraffin. Grind the sample into fine powder using liquid nitrogen.

b. Transfer the sections or ground block into the wells of the provided 96-Well Incubation Plate.
c. Add 1 mL of xylene to each sample. Seal the wells with the provided adhesive tape. Mix the sample by slight agitation.
d. Incubate at 55°C for 5 minutes.
e. Centrifuge the plate at 3,000 x g (~ 3,000 RPM) for 2 minutes.
f. Carefully remove the xylene from each well without dislodging the pellet.
g. Add 1 mL of 95 - 100 % ethanol. Mix by vortexing.
h. Centrifuge the sample at 3,000 x g (~ 3,000 RPM) for 2 minutes.
i. Carefully remove the ethanol without dislodging the pellet.
j. Repeat Step **1Ag** to Step **1Ai** for a second time.
k. Air dry the pellet for about 10 minutes at room temperature.

   Note: It is important to remove the ethanol completely.

l. Proceed to Step 2. **Lysate Preparation**

1B. Deparaffinization using microcentrifuge tubes

a. Cut sections up to 20 µm thick from the interior of an FFPE tissue block using a microtome. Trim off any excess paraffin.

   Note: Alternatively, from an FFPE block, cut out up to 25 mg of unsectioned core. Trim off any excess paraffin. Grind the sample into fine powder using liquid nitrogen.

b. Transfer the sections or ground block into an RNase-free microcentrifuge tube.
c. Add 1 mL of xylene to the sample. Mix by vortexing.
d. Incubate at 55°C for 5 minutes.
e. Centrifuge the sample at 14,000 x g (~ 14,000 RPM) for 2 minutes.
f. Carefully remove the xylene without dislodging the pellet.
g. Add 1 mL of 95 - 100 % ethanol. Mix by vortexing.
h. Centrifuge the sample at 14,000 x g (~ 14,000 RPM) for 2 minutes.
i. Carefully remove the ethanol without dislodging the pellet.
j. Repeat Step **1Bg** to Step **1Bi** for a second time.
k. Air dry the pellet for about 10 minutes at room temperature.

   Note: It is important to remove the ethanol completely.

l. Proceed to Step 2. **Lysate Preparation**

2. Lysate Preparation

a. Add 200 µL of Digestion Buffer and 10 µL of the reconstituted Proteinase K to the sample. Seal the plate with adhesive tape or close the cap of the microcentrifuge tube. Mix by agitation for samples in the 96-Well Incubation Plate or vortexing for those in microcentrifuge tubes
b. Incubate at 55°C for 15 minutes, followed by 80°C for 15 minutes. Agitate or vortex to mix occasionally for 96-Well Incubation Plate or microcentrifuge, respectively.

   Note: Do not exceed 15 minutes of incubation at 80 °C as this will increase RNA fragmentation.

   Note: For the 96-Well Incubation Plate, gently tap the plate against the bench top after the incubation to remove any condensation on top of the wells.

c. Add 200 µL of **Binding Solution**. Agitate or vortex to mix for 96-Well Incubation Plate or microcentrifuge, respectively.
d. Add 400 µL of 95 – 100 % ethanol. Agitate or vortex to mix for 96-Well Incubation Plate or microcentrifuge, respectively.

Section 2. FFPE RNA Purification

Note: The purification of total RNA from the lysate prepared in Section 1 could be performed using either a vacuum manifold or centrifugation. For purification using vacuum, please follow the procedure outlined in Section 2A. For purification using centrifugation, please follow the procedure outlined in Section 2B

A. FFPE RNA Purification Using Vacuum Manifold

3. Binding RNA to 96-Well Filter Plate

a. Assemble the 96-Well Filter Plate and the vacuum manifold according to manufacturer's recommendations.

   Note: The provided 96-Well Collection Plate can be used as the collection/waste tray if desired.

b. Apply the lysate with the ethanol (from **Step 2**) into each well of the 96-Well Filter Plate. Tape the plate or any unused wells using sealing tape or pads (provided by the user) according to the vacuum manifold manufacturer's recommendations. Apply vacuum for 2 minutes.

   Note: Depending on the starting material, a small quantity of precipitates may appear in the lysate-ethanol mix. No additional step is required to remove these precipitates prior to application of the mixture to the wells.

c. Turn off vacuum and ventilate the manifold. Discard the flowthrough. Reassemble the 96-Well Filter Plate and the vacuum manifold.

   Note: Ensure that all of the lysate from each well has passed through into the collection/waste tray. If the entire lysate volume has not passed, apply vacuum for an additional 2 minutes.

Optional Step:

Norgen's FFPE RNA Purification 96-Well Kit isolates total RNA with minimal amounts of genomic DNA contamination. However, an optional **On-Plate DNA Removal Protocol** is

provided in Appendix A for maximum removal of residual DNA that may affect sensitive downstream applications. This step should be performed at this point in the protocol.

4. RNA Wash

a. Apply 400 µL of **Wash Solution** to each well of the 96-Well Filter Plate. Tape the plate or any unused wells using sealing tape or pads (provided by the user) according to the vacuum manifold manufacturer's recommendations. Apply vacuum for 2 minutes.

Note: Ensure the entire wash solution has passed through into the collection/waste tray by inspecting the 96-Well Filter Plate. If the entire wash volume has not passed, apply vacuum for an additional 2 minutes.

b. Turn off vacuum and ventilate the manifold. Discard the flowthrough.
c. Reassemble the 96-Well Filter Plate and the vacuum manifold. Repeat steps **4a** and **4b** to wash column for a second time.
d. Reassemble the 96-Well Filter Plate and the vacuum manifold. Repeat steps **4a** and **4b** to wash column for a third time.
e. Pat the bottom of the 96-Well Filter Plate dry. Reassemble the 96-Well Filter Plate and the vacuum manifold. Apply vacuum for an additional 5 minutes in order to completely dry the plate.
f. Turn off vacuum and ventilate the manifold.

5. RNA Elution

a. Replace the collection/waste tray in the vacuum manifold with the provided 96-Well Elution Plate. Complete the vacuum manifold assembly with the 96-Well Filter Plate.
b. Add 75 µL of **Elution Solution** to each well of the plate.
c. Apply vacuum for 2 minutes.

6. Storage of RNA

Use the provided adhesive tape to seal the 96-Well Elution Plate. The purified RNA samples may be stored at –20°C for a few days. It is recommended that samples be placed at –70°C for long term storage.

B. FFPE RNA Purification Using Centrifugation

3. Binding RNA to 96-Well Filter Plate

a. Place the 96-Well Filter Plate on top of a provided 96-Well Collection Plate.
b. Apply up to 500 µL of the lysate with the ethanol (from **Step 2**) into each well of the 96-Well Filter Plate. Centrifuge the assembly at maximum speed or 3,000 *x g* (~3,000 RPM) for 2 minutes.

Note: Depending on the starting material, a small quantity of precipitates may appear in the lysate-ethanol mix. No additional step is required to remove these precipitates prior to application to the wells

c. Discard the flowthrough. Reassemble the the 96-Well Filter Plate and the bottom plate.

Note: Ensure that all of the lysate from each well has passed through into the bottom plate. If the entire lysate volume has not passed, centrifuge for an additional 2 minutes.

Optional Step:

Norgen's FFPE RNA Purification 96-Well Kit isolates total RNA with minimal amounts of genomic DNA contamination. However, an optional **On-Plate DNA Removal Protocol** is

provided in Appendix A for maximum removal of residual DNA that may affect sensitive downstream applications. This step should be performed at this point in the protocol

4. RNA Wash

a. Apply 400 µL of **Wash Solution** to each well of the 96-Well Filter Plate. Centrifuge the assembly at maximum speed or 3,000 *x g* (~3,000 RPM) for 2 minutes.

Note: Ensure the entire wash solution has passed through into the bottom plate by inspecting the 96-Well Filter Plate. If the entire wash volume has not passed, centrifuge for an additional 2 minutes.

b. Discard the flowthrough. Reassemble the 96-Well Filter Plate and the bottom plate.
c. Repeat steps **4a** and **4b** to wash column for a second time.
d. Repeat steps **4a** and **4b** to wash column for a third time.
e. Pat the bottom of the 96-Well Filter Plate dry. Reassemble the 96-Well Filter Plate and the bottom plate. Centrifuge the assembly at maximum speed or 3,000 *x g* (~3,000 RPM) for 5 minutes in order to completely dry the plate.

5. RNA Elution

a. Stack the 96-Well Filter Plate on top of the 96-Well Elution Plate.
b. Add 75 µL of **Elution Solution** to each well of the 96-Well Filter Plate.
c. Centrifuge the assembly at maximum speed or 3,000 *x g* (~3,000 RPM) for 2 minutes.

6. Storage of RNA

Use the provided adhesive tape to seal the 96-Well Elution Plate. The purified RNA sample may be stored at –20°C for a few days. It is recommended that samples be placed at –70°C for long term storage.

Appendix A

Protocol for Optional On-Column DNA Removal

Norgen's FFPE RNA Purification 96-Well Kit isolates total RNA with minimal amounts of genomic DNA contamination. However, an optional protocol is provided below for maximum removal of residual DNA that may affect sensitive downstream applications. It is recommended that Norgen's RNase-Free DNase I Kit (Product # 25710) be used for this step.

1. For every on-column reaction to be performed, prepare a mix of 5 µL of **DNase I** and 75 µL of **Enzyme Incubation Buffer** using Norgen's RNase-Free DNase I Kit (Product # 25710). Mix gently by inverting the tube a few times. **DO NOT VORTEX**.

   Note: If using an alternative DNase I, prepare a working stock of 0.25 Kunitz unit/µL RNase-free DNase I solution according to the manufacturer's instructions. A 75 µL aliquot is required for each well to be treated.

2. Perform the appropriate FFPE RNA Isolation Procedure up to and including "**Binding RNA to 96-Well Filter Plate**" (Steps 1 to 3 of all protocols)
3. ***For Vacuum Manifold*****:** Apply 400 µL of **Wash Solution** to each well of the 96-Well Filter Plate. Tape the plate or any unused wells using sealing tape or a pad (provided by the user) according to the vacuum manifold manufacturer's recommendations. Apply vacuum for 2 minutes.

   For Centrifugation**:** Apply 400 µL of **Wash Solution** to each well of the 96-Well Filter Plate. Centrifuge the assembly at maximum speed or 3,000 *x g* (~3,000 RPM) for 2 minutes.

4. Discard the flowthrough. Reassemble the 96-Well Filter Plate with the vacuum manifold or the bottom plate.
5. Apply 75 µL of the RNase-free DNase I solution prepared in Step 1 to each well of the 96-Well Filter Plate.
   ***For Vacuum Manifold*:** Apply vacuum for 30 seconds.
   ***For Centrifugation*:** Centrifuge the assembly at maximum speed or 3,000 *x g* (~3,000 RPM) for 30 seconds.
6. After the centrifugation or vacuum in Step 5, pipette the flowthrough that is present in the collection plate back onto the top of the well.

   Note: Ensure Step 6 is performed in order to ensure maximum DNase activity and to obtain maximum yields of RNA, in particular for small RNA species

7. Incubate the assembly at 25 - 30°C for 15 minutes.
8. Without any further centrifugation, proceed directly to "**RNA Wash**" Section 2A, Step 4c for **Vacuum Manifold** procedure or Section 2B, Step 4c for **Centrifugation** procedure.

Troubleshooting Guide

Problem	Possible Cause	Solution and Explanation
Poor RNA Recovery	Incomplete lysis of cells or tissue	Ensure that the appropriate amount of Digestion Buffer with Proteinase K added was used. Increase the incubation time.
	Wells has become clogged	Do not exceed the recommended amounts of starting materials. The amount of starting material may need to be decreased if the well shows clogging below the recommended levels. See also "Clogged Wells" below.
	An alternative elution solution was used	It is recommended that the Elution Solution supplied with this kit be used for maximum RNA recovery.
	Ethanol or Binding Solution was not added to the lysate	Ensure that the appropriate amount of ethanol and Binding Solution are added to the lysate before binding to the well.
	Ethanol was not added to the Wash Solution	Ensure that 50 mL of 95% ethanol is added to the supplied Wash Solution prior to use.
	Low RNA content in cells or tissues used	Different tissues and cells have different RNA contents, and thus the expected yield of RNA will vary greatly from these different sources. Please check literature to determine the expected RNA content of your starting material.

Problem	Possible Cause	Solution and Explanation
Clogged Wells	Insufficient solubilization of cells or tissues	Ensure that the appropriate amount of lysis buffer was used for the amount of cells or tissue.
	Insufficient Vacuum	Ensure that a vacuum pressure of at least -650 mbar or -25 in. Hg is developed
	Maximum number of cells or amount of tissue exceeds kit specifications	Refer to specifications to determine if amount of starting material falls within kit specifications
	Clarified lysate was not used for the binding step	Ensure that after the lysis step the sample is centrifuged if a significant amount of debris is present, and that only the clarified lysate is used in subsequent steps.
	Centrifuge temperature too low	Ensure that the centrifuge remains at room temperature throughout the procedure. Temperatures below 15°C may cause precipitates to form that can cause the wells to clog.
RNA is Degraded	FFPE sample is old	The quality of RNA purified may drastically decrease in old samples. For best performance, freshly prepared samples are highly recommended.
	RNase contamination	RNases may be introduced during the use of the kit. Ensure proper procedures are followed when working with RNA. Please refer to "*Working with RNA"* at the beginning of this user guide.
	Procedure not performed quickly enough	In order to maintain the integrity of the RNA, it is important that the procedure be performed quickly. This is especially important for the Cell Lysate Preparation Step in the Animal Tissue protocol, since the RNA in animal tissues is not protected after harvesting until it is disrupted and homogenized.
	Improper storage of the purified RNA	For short term storage RNA samples may be stored at –20°C for a few days. It is recommended that samples be stored at –70°C for longer term storage.
	Prolonged incubation at 80°C	In order to reverse formalin crosslinks, an incubation at 80°C is required. Do not exceed 15 minutes of incubation at 80°C as this will increase RNA fragmentation.
	Starting material may have a high RNase content	For starting materials with high RNAase content, it is recommended that β-mercaptoethanol be added to the Binding Solution.

Problem	Possible Cause	Solution and Explanation
RNA does not perform well in downstream applications	RNA was not washed 3 times with the provided Wash Solution	Traces of salt from the binding step may remain in the sample if the well is not washed 3 times with Wash Solution. Salt may interfere with downstream applications, and thus must be washed from the well.
	Ethanol carryover	Ensure that the dry spin under the Well Wash procedure is performed, in order to remove traces of ethanol prior to elution. Ethanol is known to interfere with many downstream applications.
	Formalin crosslink was not completely reversed	Ensure the sufficient incubation at 80°C is performed in Step **2b**. Do not exceed 15 minutes of incubation at 80°C as this will increase RNA fragmentation.

Related Products	Product #
FFPE RNA/DNA Purification Kit	25000
RNase-Free DNase I Kit	25710
RNA/Protein Purification Kit	23000
RNA/DNA/Protein Purification Kit	23500
microRNA Purification Kit	21300
100b RNA Ladder	15002
1kb RNA Ladder	15003

Technical Support
Contact our Technical Support Team between the hours of 8:30 and 5:30 (Eastern Standard Time) at (905) 227-8848 or Toll Free at 1-866-667-4362.
Technical support can also be obtained from our website (www.norgenbiotek.com) or through email at techsupport@norgenbiotek.com.

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